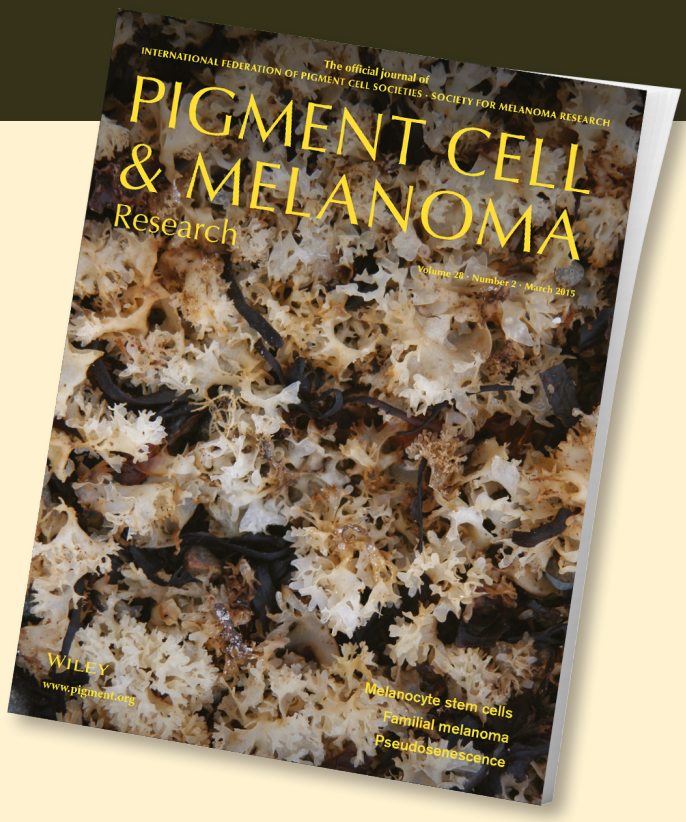
Temperature-sensitive splicing of mitfa by an intron mutation in zebrafish

The official journal of
INTERNATIONAL FEDERATION OF PIGMENT CELL SOCIETIES · SOCIETY FOR MELANOMA RESEARCH
PIGMENT CELL & MELANOMA Research



Temperature-sensitive splicing of *mitfa* by an intron mutation in zebrafish

Zhiqiang Zeng, Stephen L. Johnson, James A. Lister and E. Elizabeth Patton

DOI: 10.1111/pcmr.12336
Volume 28, Issue 2, Pages 229–232

Temperature-sensitive splicing of *mitfa* by an intron mutation in zebrafish

Zhiqiang Zeng[1], Stephen L. Johnson[2], James A. Lister[3] and E. Elizabeth Patton[1]

1 MRC IGMM, MRC Human Genetics Unit, University of Edinburgh Cancer Research UK Centre, Edinburgh, UK **2** Department of Genetics, Washington University School of Medicine, St. Louis, Missouri, USA **3** Department of Human and Molecular Genetics, Virginia Commonwealth University School of Medicine, Richmond, Virginia, USA
Correspondence James A. Lister and E. Elizabeth Patton, e-mails: jalister@vcu.edu and e.patton@igmm.ed.ac.uk

doi: 10.1111/pcmr.12336

Dear Editor,

Temperature-sensitive (*ts*) mutations have provided fundamental insight into our understanding of gene function in vivo. Although they are rare in higher organisms, temperature-sensitive mutations in the pigmentation pathways include *tyrosinase ts* mutations found in oculocutaneous albinism (OMIM: 606952), in Siamese cats and the Himalayan mouse (Giebel et al., 1991), and in *kit* in zebrafish (Rawls and Johnson, 2001). Microphthalmia-associated transcription factor (MITF) is the master melanocyte transcription factor that has critical functions in melanocyte development, melanocyte stem cell renewal, and the tanning response, and is an important drug target (Hsiao and Fisher, 2014). Given the importance of MITF, it is critical to develop laboratory animals that enable conditional control of MITF activity in different temporal, cellular, developmental, and disease contexts. Conditional or *ts* alleles in *MITF* have not been described in mammals. We (SLJ, JAL) have previously identified the first conditional *MITF* mutation (*mitfa*^{vc7}) in an animal in an ENU-based genetic screen in zebrafish, but the molecular mechanism underlying this mutation was unknown (Johnson et al., 2011). Here, we explain the molecular mechanism that enables the *mitfa*^{vc7} mutation to confer conditional temperature-dependent control of MITF activity in zebrafish, and describe an unusual intron mutation that leads to aberrant splicing of wild-type and dominant negative splice variants at the restrictive temperatures.

Most temperature-sensitive mutations affect exon sequences, but the *mitfa*^{vc7} mutation is unusual because it is in an intron splice donor (t>a at position 39638 in *mitfa* genomic locus or position 2 in intron 6; Johnson et al., 2011). We collected wild-type and *mitfa*^{vc7} mutant zebrafish embryos and grew the animals at 24, 26, 28.5, and 32°C. At higher temperatures, zebrafish embryos grow more rapidly, and to compensate for this, for all experiments, zebrafish embryos were carefully stage-matched. At 32°C, *mitfa*^{vc7} zebrafish embryos lack all neural crest-derived melanocytes due to loss of *mitfa* activity (Figure 1A). At 24°C, wild-type and *mitfa*^{vc7} zebrafish embryos develop similar numbers of clearly visible and pigmented melanocytes (Johnson et al., 2011), albeit with a delay in differentiation and/or cell size in the *mitfa*^{vc7} mutant embryos at 2 days post-fertilization (Figure 1A). We examined the *mitfa* RNA encompassing exons 4–7 and found correct splicing occurs at low (permissive) temperatures along with aberrant splicing, while aberrant splice forms predominate with increasing temperature (Figure 1B; as described in the Appendix S1). Temperature-sensitive splicing at this locus had not previously been detected (Johnson et al., 2011), possibly due to PCR conditions. We cloned each of the splice forms (labeled a–c) and found that the aberrant splice forms include those that skip exons or retain introns (Figure 1C). Splice form 'a' includes introns 5 and 6 (*mitfa*$^{+in5,6}$), form 'b' includes an in-frame intron 6 (*mitfa*$^{+in6}$), and splice form 'c' leads to an in-frame deletion of exon 6 (*mitfa*$^{\Delta ex6}$). At the highest temperature (32°C), almost no wild-type *mitfa* or *mitfa*$^{+in6}$ was detectable, while there was strong expression of *mitfa*$^{\Delta ex6}$. These results demonstrate that aberrant of the *mitfa* RNA correlates with the temperature sensitivity of the *mitfa*^{vc7} mutation.

Aberrant splice variants can have partial or neomorphic function. To establish whether the splice variants had activity, we cloned the splice variants under the control of the *mitfa* promoter and microinjected them into zebrafish null *mitfa* (the *mitfa*^{w2} (*nacre*) mutation, a premature stop in exon 3, in which there are no neural crest-derived melanocytes; Lister et al. 1999; Figure 1C). Expression of wild-type *mitfa* rescued the *nacre* mutation, and melanocytes were clearly visible at 5 dpf. In contrast, the *mitfa*$^{\Delta ex6}$ isoform was unable to stimulate melanocyte development in *nacre* mutants. The *mitfa*$^{+in6}$ splice variant was functional in this assay, albeit with significantly reduced activity compared to wild-type *mitfa*. In addition, the *mitfa*$^{+in5,6}$ isoform demonstrated significantly reduced activity compared to *mitfa* and compared to *mitfa*$^{+in6}$. At 32°C, *mitfa*$^{+in6}$ was significantly reduced in its activity and *mitfa*$^{+in5,6}$ had no function at all, demonstrating the temperature sensitivity of the splice products.

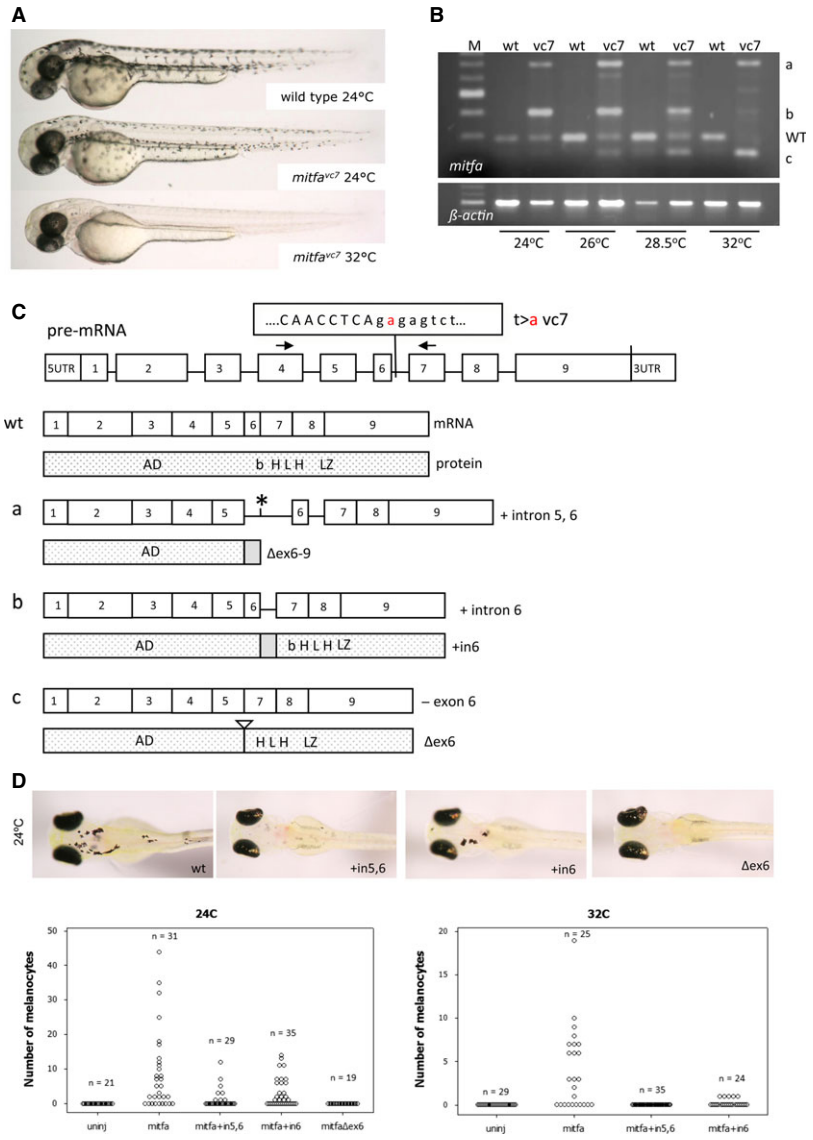


Figure 1. The *mitfa*^{vc7} intron 6 mutation causes defective temperature-dependent splicing of *mitfa*. (A) Images of *mitfa*^{vc7} zebrafish embryos (2 days post-fertilization) at 24°C (permissive temperature) and 32°C (restrictive temperature). Pigmented melanocytes are clearly visible on the body of the zebrafish. (B) RT-PCR *mitfa* RNA expression at 24, 26, 28.5, and 32°C in wild-type and *mitfa*^{vc7} mutant embryos. Four types of *mitfa* transcripts (wt, a, b, c) were consistently observed in *mitfa*^{vc7} embryo across all temperatures, while only one transcript (wt) was observed in wild-type embryo. (C) Schematic overview of *mitfa*^{vc7} splice variant transcripts and their predicted MITF protein products. (D) Images and quantitation of zebrafish embryos following injection with transgenes expressing the *mitfa*^{vc7} splice variants from the *mitfa* promoter at 24 and 32°C. At 24°C, the number of melanocytes promoted by expression of the *mitfa*$^{+in6}$ splice variant [2.97 (2.27–3.67); mean (95% CI)] was significantly less active compared to wild-type *mitfa* [8.71 (6.66–10.76); mean (95% CI); P = 0.007; ANOVA]. In addition, the number of melanocytes promoted by expression of *mitfa*$^{+in5,6}$ [1.138 (0.64–1.636); mean (95% CI)] was significantly less than *mitfa* (P = 0.001; ANOVA) and compared to *mitfa*$^{+in6}$ (P = 0.045; ANOVA).

Given the activity of *mitfa*$^{+in6}$, and the very weak activity of *mitfa*$^{+in5,6}$, we hypothesized that some *mitfa* pre-RNA species can be correctly spliced in these transcripts to wild-type *mitfa* and promote melanocyte development. To explore this idea, we examined the *mitfa* transcripts in the *mitfa*$^{+in6}$ embryos at 24 and 28°C and found transcripts that were the same size as wild-type *mitfa*. However, the interpretation of this was complicated by the presence of *mitfa*^{nacre} transcripts that are also the same size as wild-type *mitfa* (502 bp; Figure 2A). Sequencing the 502-bp species in the *mitfa*$^{+in6}$-expressing *nacre* mutant embryos revealed both the *nacre* and wild-type *mitfa* transcripts indicating that *mitfa*$^{+in6}$ splice variants could be correctly spliced to the wild-type form in the *nacre* embryos (Figure 2B). While not tested, we anticipate a similar mechanism explains the few melanocytes that develop in the *mitfa*$^{+in5,6}$-expressing *nacre* animals, rather than functional Mitfa activity of the protein product of the splice variant, because the encoded protein for the *mitfa*$^{\Delta ex6-9}$ lacks the DNA binding and dimerization domains (Figure 1C, D). Subsequent PCR analysis suggests that the reduction of the *mitfa*^{nacre} transcript in the *mitfa*$^{+in6}$-injected embryos (Figure 2A) is due to PCR template competition in favor of the ectopically expressed *mitfa*$^{+in6}$, rather than regulation of *mitfa*^{nacre} expression by Mitfa^{+In6} (data not shown).

Next, we altered the splice acceptor and donor sites to test whether the *mitfa*$^{+in6}$ splice variants can have activity independently of splicing to the wild-type form (Figure 2C–E). Restoration of the splice donor site (ga>gt) in the *mitfa*$^{+in6}$ cDNA was sufficient to restore Mitf activity in the transgene, while change to another nucleotide (ga>gg) resulted in further reduced activity (Figure 2D). In contrast, when we maintained the *mitfa*$^{+in6}$ mutation, but destroyed the 3′ splice acceptor site (ag>tg), it was non-functional in the *nacre* mutants (Figure 2E). Taken together, these

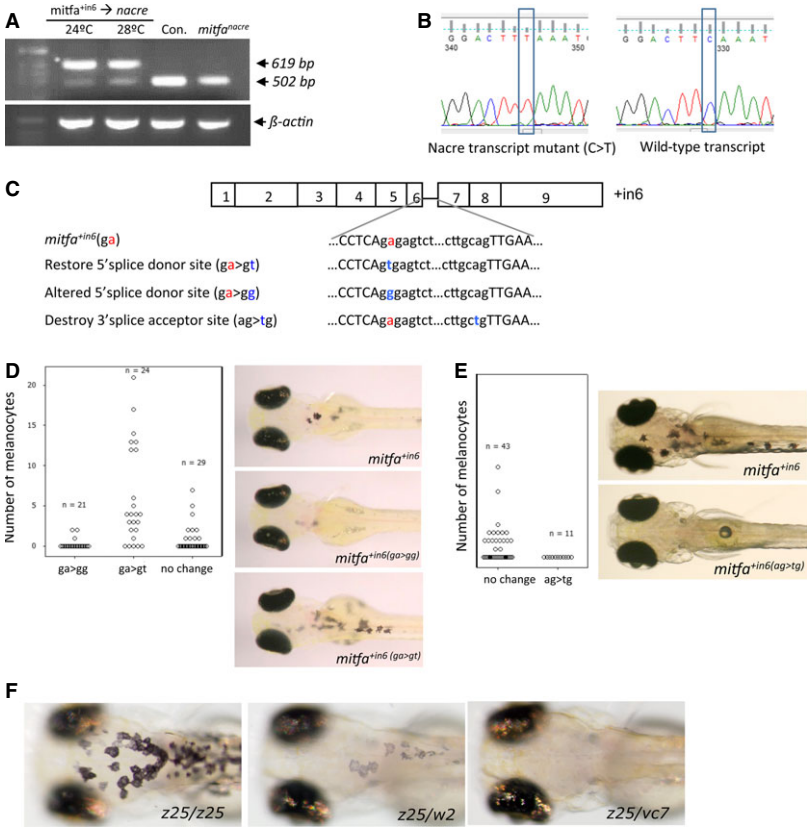


Figure 2. *mitfa*^{vc7} temperature sensitivity is determined by reduction of wild-type *mitfa* mRNA coupled with dominant negative activity of novel splice variants. (A) RT-PCR analysis of *mitfa* in *nacre* mutant zebrafish expressing the *mitfa*$^{+in6}$ transgene and in control wild-type and uninjected *nacre* mutant zebrafish. The correctly spliced *mitfa* (502 bp) and the *mitfa*$^{+in6}$ variant (619 bp) are indicated. (B) Sequencing traces of the correctly spliced *mitfa* PCR product identified in A. Cloned transcripts contained the *nacre* transcript with a mutation C>T and wild-type *mitfa* correctly spliced from the *mitfa*$^{+in6}$ transcript. (C) Illustration of the *mitfa*$^{+in6}$ 5′ splice donor site and the 3′ splice acceptor site, and engineered mutations. (D, E) Quantitation and images of *nacre* zebrafish embryos: (D) expressing the *mitfa*$^{+in6}$ transgene with the restored 5′ donor site (A>T), the altered 5′ donor site (A>G), (E) and when the 3′ splice acceptor site is destroyed. (F) Images of *mitfa* mutant zebrafish embryos at 32°C. *mitfa*^{z25} is a hypomorphic allele that results in fewer and pale melanocytes as *mitfa*$^{z25/w2}$, while no body melanocytes are present in *mitfa*$^{vc7/z25}$.

experiments indicate that the *mitfa*$^{+in6}$ can be spliced to the wild-type *mitfa* form and that the activity of *mitfa*$^{+in6}$ is due to the minor accumulation of the wild-type species. The particular mutation (t>a) appears to be crucial to the unique temperature-sensitive splicing.

MITF binds to DNA as a homodimer, and the aberrant splice variants could interfere with wild-type MITF activity. Deletion of exon 6 is predicted to truncate the basic region (Figure 1C). The dominant negative alleles in mouse cluster in the basic region, which is necessary for DNA binding, while retaining dimerization capability (reviewed in Steingrímsson et al., 2004). Heterozygous *mitfa*^{vc7} mutants appear similar to wild-type zebrafish at 5 dpf (Johnson et al., 2011), suggesting that the dominant negative activity of the *vc7* mutation is not sufficient to produce a robust phenotype by interfering with wild-type protein. However, when combined with the weakly active *mitfa*^{z25} mutation (a substitution, I219F, in the first helix of the HLH domain; Johnson et al., 2011), the *mitfa*$^{z25/vc7}$ mutant embryos failed to develop melanocytes at 32°C, while the *mitfa*$^{z25/w2}$ mutant embryos were able to develop a few and weakly pigmented melanocytes (Figure 2F). These results indicate that the *mitfa*^{vc7} splice variants have some weak dominant interfering activity that may contribute to complete loss of *mitfa* activity at 32°C.

Temperature-sensitive mutations are classic genetic tools that enable functional and temporal control of gene action. Our zebrafish *mitfa*^{vc7} temperature-sensitive mutant is the only conditional MITF mutation in vertebrates and has already provided insight into the function of MITF in melanocyte stem cells (Johnson et al., 2011), melanocyte development and differentiation (Johnson et al., 2011; Taylor et al., 2011), and in melanoma (Lister et al., 2014). Unusually, rather than increasing temperature affecting the protein function directly as is the case with most temperature-sensitive mutations, *mitfa*^{vc7} is an intron mutation that leads to aberrant splice forms. Few examples of temperature-sensitive splicing due to intron mutations are found in the literature. In human disease, temperature-dependent splicing in ß-globin pre-mRNA of patients with thalassemia is caused by a mutation in intron 2 (Gemignani et al., 2002), and temperature-sensitive aberrant splicing of type III procollagen transcripts is caused by mutations in splice donor sites within introns in patients with Ehler-Danlos syndrome type IV (Lee et al., 1991; and references therein). In *Arabidopsis*, an exon mutation close to a 5′-splice site confers temperature sensitivity of RNA splicing in the floral homeotic gene APETALA3 (called the *ap3-1* mutant) that controls stamen and petal development (Sablowski and Meyerowitz, 1998). Conditional mutants have been cleverly engineered by the addition of temperature-sensitive DEGRON (Dohmen et al., 1994) and self-excising intein (excising protein) sequences (Tan et al., 2009; Zeidler et al., 2004). We have tested whether the intron 6 can confer temperature sensitivity to GFP in

zebrafish but have thus far been unsuccessful: additional exonic sequences may be required to enable temperature-sensitive splicing to be engineered into other genes.

To conclude, our work explains the temperature sensitivity of the *mitfa*^{vc7} mutation to be due to an unusual intron 6 mutation that leads to reduced levels of wild-type *mitfa* RNA. The production of interfering variants may also ensure that no melanocytes develop at 32°C. We suggest that the *mitfa*^{vc7} mutation compromises base pairing with the small nuclear RNAs of the spliceosome and that this interaction becomes destabilized at the restrictive temperatures. Alternative splicing is an integral feature of *MITF* pre-RNA processing and gives rise to multiple MITF spice variants that are both melanocyte specific and relevant in melanocyte development and melanoma (Bharti et al., 2010; Cronin et al., 2009; Debbache et al., 2012; Simmons et al., 2014). Given the importance of MITF in melanocytes, the *mitfa*^{vc7} allele enables careful examination of MITF activity at multiple stages of melanocyte development, stem cells and melanoma, and is a unique means to explore the function of aberrant pre-RNA splicing in zebrafish.

Acknowledgements

We are grateful to Professor Ian Jackson for many helpful discussions and to Dr. Karthika Paranthaman and Wei Qing for zebrafish husbandry. This work was funded by the NIH (to SLJ, Grant Number RO1GM056988), the MRC (to ZZ, EEP), and Concern Foundation for Cancer Research (to JAL).

References

Bharti, K., Debbache, J., Wang, X., and Arnheiter, H. (2010). The basic-helix–loop–helix–leucine zipper gene Mitf: analysis of alternative promoter choice and splicing. Methods Mol. Biol. *647*, 237–250.

Cronin, J.C., Wunderlich, J., Loftus, S.K. et al. (2009). Frequent mutations in the MITF pathway in melanoma. Pigment Cell Melanoma Res. *22*, 435–444.

Debbache, J., Zaidi, M.R., Davis, S. et al. (2012). In vivo role of alternative splicing and serine phosphorylation of the microphthalmia-associated transcription factor. Genetics *191*, 133–144.

Dohmen, R.J., Wu, P., and Varshavsky, A. (1994). Heat-inducible degron: a method for constructing temperature-sensitive mutants. Science *263*, 1273–1276.

Gemignani, F., Sazani, P., Morcos, P., and Kole, R. (2002). Temperature-dependent splicing of beta-globin pre-mRNA. Nucleic Acids Res. *30*, 4592–4598.

Giebel, L.B., Tripathi, R.K., King, R.A., and Spritz, R.A. (1991). A tyrosinase gene missense mutation in temperature-sensitive type I oculocutaneous albinism. A human homologue to the Siamese cat and the Himalayan mouse. J. Clin. Invest. *87*, 1119–1122.

Hsiao, J.J., and Fisher, D.E. (2014). The roles of microphthalmia-associated transcription factor and pigmentation in melanoma. Arch. Biochem. Biophys. *563C*, 28–34.

Johnson, S.L., Nguyen, A.N., and Lister, J.A. (2011). mitfa is required at multiple stages of melanocyte differentiation but not to establish the melanocyte stem cell. Dev. Biol. *350*, 405–413.

Lee, B., Vitale, E., Superti-Furga, A., Steinmann, B., and Ramirez, F. (1991). G to T transversion at position +5 of a splice donor site causes skipping of the preceding exon in the type III procollagen transcripts of a patient with Ehlers–Danlos syndrome type IV. J. Biol. Chem. *266*, 5256–5259.

Lister, J.A., Robertson, C.P., Lepage, T., Johnson, S.L., and Raible, D.W. (1999). nacre encodes a zebrafish microphthalmia-related protein that regulates neural-crest-derived pigment cell fate. Development. *126*, 3757–3767.

Lister, J.A., Capper, A., Zeng, Z., Mathers, M.E., Richardson, J., Paranthaman, K., Jackson, I.J., and Patton, E.E. (2014). A conditional zebrafish MITF mutation reveals MITF levels are critical for melanoma promotion vs. regression in vivo. J. Invest. Dermatol. *134*, 133–140.

Rawls, J.F., and Johnson, S.L. (2001). Requirements for the kit receptor tyrosine kinase during regeneration of zebrafish fin melanocytes. Development *128*, 1943–1949.

Sablowski, R.W., and Meyerowitz, E.M. (1998). Temperature-sensitive splicing in the floral homeotic mutant apetala3-1. Plant Cell *10*, 1453–1463.

Simmons, J.L., Pierce, C.J., and Boyle, G.M. (2014). Evidence for an alternatively spliced MITF exon 2 variant. J. Invest. Dermatol. *134*, 1166–1168.

Steingrímsson, E., Copeland, N.G., and Jenkins, N.A. (2004). Melanocytes and the microphthalmia transcription factor network. Annu. Rev. Genet. *38*, 365–411.

Tan, G., Chen, M., Foote, C., and Tan, C. (2009). Temperature-sensitive mutations made easy: generating conditional mutations by using temperature-sensitive inteins that function within different temperature ranges. Genetics *183*, 13–22.

Taylor, K.L., Lister, J.A., Zeng, Z., Ishizaki, H., Anderson, C., Kelsh, R.N., Jackson, I.J., and Patton, E.E. (2011). Differentiated melanocyte cell division occurs in vivo and is promoted by mutations in Mitf. Development *138*, 3579–3589.

Zeidler, M.P., Tan, C., Bellaiche, Y., Cherry, S., Hader, S., Gayko, U., and Perrimon, N. (2004). Temperature-sensitive control of protein activity by conditionally splicing inteins. Nat. Biotechnol. *22*, 871–876.

Supporting information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Methods